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Drug-induced injury due to flucloxacillin: relevance of multiple HLA alleles

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Abstract

Some patients prescribed flucloxacillin (~0.01%) develop drug-induced liver injury (DILI). *HLA-B*57:01* is an established genetic risk factor for flucloxacillin DILI. To consolidate this finding, identify additional genetic factors and assess relevance of risk factors for flucloxacillin DILI in relation to DILI due to other penicillins, we performed a genome-wide association study involving 197 flucloxacillin DILI cases and 6835 controls. We imputed SNP and HLA genotypes. *HLA-B*57:01* was the major risk factor (allelic OR=36.62, P=2.67x10^{-97}). *HLA-B*57:03* also showed an association (OR=79.21, P=1.2x10^{-6}). Within the HLA-B protein sequence, imputation showed valine^{97}, common to HLA-B*57:01 and HLA-B*57:03, had the largest effect (OR=38.1, P=9.7x10^{-97}). We found no *HLA-B*57* association with DILI due to other isoxazolyl penicillins (n=6) or amoxicillin (n=15) and no significant non-HLA signals for any penicillin-related DILI. (125 words)

Flucloxacillin is a very common cause of drug-induced liver injury (DILI) in a number of countries worldwide, especially in Northern Europe and Australasia.(1) The incidence of DILI due to this drug is 8.5 per 100,000 people prescribed flucloxacillin increasing to 35 per 100,000 in those receiving one consecutive prescription and 110.5 per 100,000 in those aged >70 years who have received two or more prescriptions.(2) Most patients recover completely but some may develop chronic disease with effects such as vanishing bile duct syndrome.(1) The toxicity is occasionally fatal.(3) Though flucloxacillin DILI was originally thought to be due to impaired drug metabolism (4), features such as rash and fever more typical of hypersensitivity reactions are often seen.(1, 5) The relevance of the immune system to this form of adverse drug reaction was indicated by the finding that 85% of a case cohort (n=51) were positive for *HLA-B*57:01.*(6) Further studies showed that CD8-positive T cells from *HLA-B*57:01*-positive donors could be activated by flucloxacillin in a mechanism that appeared to involve the formation of drug-protein conjugates,(7) though a non-hapten mechanism has also been proposed.(8) Although carriage of *HLA-B*57:01* is also a strong risk factor for abacavir hypersensitivity, the predictive power of *B*57:01* for this adverse drug reaction is higher than for flucloxacillin-related DILI and the underlying mechanism, which involves a change in the peptide repertoire in the peptide binding grove of B*57:01 appears different.(9)

Despite the strong association of flucloxacillin DILI with *HLA-B*57:01*, overall sensitivity and specificity of genotyping to predict susceptible individuals are both too low to justify using this test routinely prior to prescription since only approx. 1 in every 500 *HLA-B*57:01* carriers, who comprise approx. 5% of Europeans, are likely to develop DILI if treated with flucloxacillin.(6) However, this form of DILI is more common than estimated previously with individuals over 70 years particularly at risk, with the number needed to test for *B*57:01* in this group to prevent one case now estimated at 2512.(2) Finding additional genetic risk factors might lower this number further. To investigate this possibility both in those positive for *B*57:01* and those who don't carry the genotype, we have extended the size of our case group and performed a further genome-wide association study (GWAS) with improved imputation of genotypes together with additional HLA typing. We have also investigated whether *B*57:01* is relevant to the risk of DILI due to the other related isoxazolyl penicillins including cloxacillin and dicloxacillin, which are often used as alternatives to flucloxacillin and are generally considered to be less important causes of DILI. We assess possible overlap in genetic risk factors for DILI due to other beta lactam antibiotics by studies on amoxicillin-related DILI.(5)

Results

Characteristics of the study subjects

The flucloxacillin DILI cases in the study were from two separate recruitment phases. Phase I consisted of cases included in two previous studies (all from UK DILIGEN study) (n=75)(6, 10) and phase II more recently recruited cases for the iDILIC study (n=122) from UK, Sweden, Netherlands and Australia. The majority of the phase I cases (n=51) were included in an earlier GWAS on flucloxacillin DILI.(6) The combined 197 cases were from the UK (n=156), Sweden (n=37), the Netherlands (n=3) and Australia (n=1). Clinical inclusion criteria for all cases were those described by Aithal et al.(11) Causality assessment and inclusion criteria were as described previously.(12) Clinical data for the flucloxacillin DILI cases (phase I and phase II) included in the GWAS are summarized in Table 1. Thirty percent of the cases (n=59) were aged 70 years or over and 68% (n=134) were female.

DILI cases relating to dicloxacillin (n=2), cloxacillin (n=2), and oxacillin (n=2) were recruited in USA and Iceland from the DILIN and iDILIC studies.(10, 12) Cases of DILI relating to amoxicillin alone were also recruited from a range of European centres (n=13) and from USA (n=2) as described previously.(12) In addition, two DILI cases from Spain relating to cloxacillin were analyzed. Data for the cases due to other isoxazolyl penicillins (n=6) and the amoxicillin only cases (n=15) are summarized in Table S1.

Since DILI has a very low prevalence, we used general population samples as study controls. Flucloxacillin cases were then compared with a total of 6835 northern European ancestry controls from multiple available sources: Wellcome Trust Case Control Consortium,(13) the population reference sample (POPRES)(14) and PGX40001.(15) In order to increase the case/control ratio for Swedish cases, we added samples from the Swedish Twin Registry.(16) For other penicillins, we compared drug-specific cases to a total of 10588 European controls reported previously which included the 6835 northern European ancestry controls described above.(12)

GWAS and HLA analysis on flucloxacillin cases

Following the initial GWAS, imputation to assign SNP and HLA genotypes was performed. Imputation methods were described in detail previously.(12) For HLA, four digit HLA alleles and amino acid sequences were inferred using SNP2HLA.(17)

Principal component analysis of the imputed SNP data confirmed the self-reported ethnicity for the cases and divided the controls into two major clusters which represent mainly UK and Swedish cases respectively (Figure 1). The GWAS results for flucloxacillin DILI are summarized in Figures 2 and S1. The only genome-wide significant peak was on chromosome 6 in the MHC region with the top SNP rs2395029 (Odds ratio (OR) = 35.48; 95% CI 25.38-49.58; P = 5.7×10^{-97}). Considering only newly recruited phase II cases (n=146), the rs2395029 association showed equivalent OR and AF (OR=31, 95%CI 22.28-45.86, P=6.9×10^{-79}, allele frequency (AF) of 40% in cases) to the OR and AF from our smaller original phase I study.(6) A number of other SNPs in the MHC region were also genome-wide significant. The genotype data for all cases was used to impute HLA alleles. In line with the GWAS findings, the strongest imputed HLA association was with *HLA-B*57:01* (OR = 36.62; 95% CI 26.14-51.29; P = 2.67×10^{-97}) followed by *HLA-C*06:02*, *HLA-DQB1*03:03*, *HLA-DRB1*07:01, HLA-DQA1*02:01* and *HLA-A*01:01* which together form part of the most common B57 haplotype (allelefrequencies.net). A second group of alleles, *HLA-C*07:02*, *HLA-B*07:02* and

*HLA-DQB1*03:01* were protective and showed p values <10^{-5} (Table 2). Haplotype analysis showed that *HLA-B*57:01*-containing haplotypes confer risk while *B*57:01*-negative haplotypes seem to be protective, suggesting that *B*57:01* and no other alleles within the haplotype is the main risk factor (Table S2).

Reciprocal conditional analyses on rs2395029 and *HLA-B*57:01* demonstrated that *HLA-B*57:03* was a MHC-significant independent risk allele (OR = 79.21; 95% CI 13.57-462.4; P = 1.2×10^{-6}). *HLA-B*57:03* is rare in Caucasians (AF = 0.0003) (Table 2). In our case group, two samples were predicted to be positive for this allele from the imputation; this was confirmed for one of the two cases by direct HLA typing. The positive *HLA-B*57:03* cases were in both the ancestry clusters. There was no difference in AF between two ancestry clusters (UK and Sweden) for *HLA-B*57:01* alleles (AF_{sweden}= 0.03 vs AF_{uk}=0.04, p-value for direct comparison between the clusters = 0.4) and *HLA-B*57:03* (AF_{sweden}= 0.0002 vs AF_{uk}= 0.0003, p-value for direct comparison between the clusters = 0.9) .

The GWAS was repeated to include the B*57-carriers only (n=163) in the case group but with the same control group as used in the main analysis. We detected no additional genome-wide significant signals in this further analysis (data not shown).

We also analyzed polymorphic amino acid residues in the HLA proteins to assess their individual contribution to flucloxacillin DILI susceptibility. Valine at position 97 (V^{97}) in the HLA-B protein (OR = 38.1, 95% CI 27.07-53.62, P = 9.7×10^{-97}) had a stronger association compared to the two significant single HLA B*57 alleles (Table 3) because V^{97} is shared by HLA-B*57:01 and HLA-B*57:03 (Figure S2). This amino acid is also shared by other B*57 alleles (16 in total) and non-B*57 alleles (6 alleles).(18) Other than for B*57, the V^{97}-associated alleles are extremely rare or not present in Caucasian populations. A complete list of the alleles and their frequency in Caucasians is shown in Table S3. Except for *HLA-B*57:03*, the cases did not carry any rare alleles positive for V^{97}. Six different amino acid variants at position 97 of HLA-B are known. Except for valine, all had a protective effect against flucloxacillin DILI, but only arginine (R)[97] and serine (S)[97] showed significant protection (OR= 0.43, P= $5.13x10^{-14}$ and OR= 0.53, P=$9.82x10^{-7}$) (Table 3).

Relationship between genetic and clinical risk factors for flucloxacillin DILI

We analyzed the contribution of age and gender to DILI risk in the cases and a subset of controls with the necessary data on age and sex (380 controls (POPRES) (14)). Although, sex, age and HLA-B*57 were significant in an univariate model, in a multivariate analysis model only age above 70 years and HLA-B*57 remained significantly associated with the DILI (Table S4). These data suggest that for those older than 70 years there was an 7 fold increase in DILI risk.

We also compared selected clinical characteristics of the cases positive for any B*57 allele with those entirely negative but there appeared to be no significant difference for any of the characteristics examined (Table S5). The proportion of patients aged 70 or over in the *HLA-B*57:01*-negative cases was compared with the positive group. Among the *B*57:01*-negative cases, 22% were in this older age group compared with 32% of those positive for *B*57:01* but this difference was not statistically significant (p=0.32).

HLA genotypes in B*57-negative flucloxacillin DILI cases

To determine whether additional HLA alleles were risk factors for DILI in this set of 34 cases (17.25% of all cases) that were negative for rs2395029, *HLA-B*57:01* and *HLA-B*57:03*, the imputed HLA genotypes for the group were analyzed separately against a negative *HLA-B*57* alleles control set (n= 6321). While no strong risk factors emerged, it was found that the rare allele *HLA-A*02:02* (0.001 in controls) was enriched in the negative cases (OR = 15.24, 95% CI 1.89-123.1, P = 0.01) but absent in the group of HLA-B*57-carriers. Two other class I alleles, *HLA-A*30:01* and *HLA-B*13:02*, showed nominally significant associations (Table 4). *HLA-B*07:02* also showed a protective effect in this group, similar to the *B*57:01*-positive group. When amino acid analysis on HLA proteins was performed on these B*57-negative cases, we found that the most associated variants were deletions of the first 30 and last 58 amino acids of the HLA-A protein (Table S6). The deletions showed a higher effect size and significance compared to the single top HLA-A alleles (OR = 19.67, 95% CI = 2.35-164.4, P = 0.006). This suggests that the short *A*02:02* alleles (including *A*02:02:02, A*02:02:04* and *A*02:02:05*) as well as some short *A*30:01* alleles (such as *A*30:01:03, A*30:01:04, A*30:01:05, A*30:01:06, A*30:01:07, A*30:01:10*) might be enriched in *HLA-B*57:01* negative cases. No samples were positive for the B*57-related V^{97}.

GWAS and HLA analysis on cases due to other penicillins

A total of 21 additional European penicillin DILI cases were available to us: 15 samples due to amoxicillin only (not amoxicillin-clavulanate) and 6 due to dicloxacillin, cloxacillin or oxacillin (Table S1). The majority of these cases (62%) had a cholestatic or mixed phenotype but the percentage was slightly lower than the 81% seen in the flucloxacillin DILI cases. As for the flucloxacillin DILI cases, GWAS analysis (Figures S3 to S5) and HLA imputation were performed. In both groups, the effect of *HLA-B*57:01* was not significant though there was a trend in the direction of a positive association (Table S7). Considering non-flucloxacillin isoxazolyl penicillin cases, the uncommon *HLA-C*07:04* and the corresponding amino acid (phenylalanine $(F)^{95}$) showed associations just above the threshold for MHC significance (both showing OR = 12.97, P= 0.001, Table 5 and Table S8). The amoxicillin DILI cases showed a significant association with *HLA-B*58:01* (OR= 20.29, 95%CI 4.25-96.94, P= 0.0002) and borderline associations with *HLA-DPB1*01:01*, *HLA-A*01:01* and *HLA-C*03:02* (Table 5). The top amino acid association was with methionine $(M)^{67}$ or F^{67} as B pocket residues of the HLA-B gene product but this was not significant (OR=3.55, 95%CI 1.69-7.40, P=0.0008). No other genetic variants were found to be genome-wide or MHC significant when the penicillin cases were analyzed, either together conditioning for *HLA-B*57* alleles or by individual drug.

Two additional cloxacillin DILI cases from Spain were not included in the main GWAS analysis which was confined to Northern Europeans, but these samples were typed directly for HLA-B alleles.

Neither was positive for *B*57:01*; the HLA-B genotypes were *B*07:02/B*44:02* and *B*08:01/B*35:02*.

Discussion

This study has confirmed that *HLA-B*57:01* is an important risk factor for DILI relating to flucloxacillin. By repeating the original analysis in a larger cohort, the p value for this risk factor was lowered but also no evidence for signals outside the chromosome 6 region was obtained. The previous GWAS had found a contribution by a SNP in *ST6GAL1* which was of borderline significance when analysis was performed using cases carrying *HLA-B*57:01* only,(6) but this was not confirmed in the current study with a substantially larger group of patients, even when B*57-carrier only analysis was performed. Improved ability to impute HLA genotypes as well as an increase in numbers of cases and some additional direct HLA typing indicated that in addition to *HLA-B*57:01, HLA-B*57:03* is also a risk factor for flucloxacillin DILI. *B*57:03* is very rare among Northern Europeans (for example estimated allele frequencies of 0 (Northern Ireland), 0.002 (Irish Republic) and 0.0007 (Germany) (www.allelefrequencies.net)). This low allele frequency makes meaningful comparisons challenging but suggests that the *HLA-B*57:01* association for flucloxacillin DILI is less specific than the association between *HLA-B*57:01* and abacavir hypersensitivity. Based mainly on studies *in vitro,* alleles related closely to *HLA-B*57:01* such as both *HLA-B*57:02* and *HLA-B*57:03* are not risk factors for abacavir hypersensitivity.(9) In view of a recent report involving an African population which reports that *HLA-B*57:02* and *HLA-B*57:03* are risk factors for an unusual form of DILI seen when both antiretroviral and anti-TB drugs are prescribed(19) as well as the strong homology in the amino acid sequence for the gene product, it is also possible that *HLA-B*57:02* and other rare B*57 alleles may be risk factors for flucloxacillin DILI. *HLA-B*57:02*, like *HLA-B*57:03*, is very rare in Northern Europeans and was not detected at all in our population but does code for V^{97} in the HLA-B gene product similar to both *HLA-B*57:01* and *HLA-B*57:03*. While R^{97} is the most common residue, encoded by a number of different common *HLA-B* alleles,(20) S^{97} is a characteristic residue for the protective *HLA-B*07:02,* which is the main flucloxacillin DILI-associated protective allele (see Table 2). The residues methionine, isoleucine and tryptophan which are of similar hydrophobicity to valine, are also found in position 97 in other HLA class I proteins. When HLA-A proteins sharing these hydrophobic residues at position 97 are considered, the alleles *HLA-A*31:01*, *A*33:01* and *A*33*03*, which are strong predictors of susceptibility to skin rash or DILI due to various drugs (12, 21), each encode M^{97}. This suggests this position and the nature of the amino acid present may be of importance in presentation of modified peptides for both HLA-A and HLA-B.

In addition to V^{97}, D^{114} and S^{116} are considered to be important in the interaction between abacavir and the B*57:01 gene product.(9) As summarised in Figure S2, amino acids 114 and 116 are not conserved in the B*57:02 and B*57:03 proteins. This finding of an apparent difference in HLA genotype selectivity for flucloxacillin DILI compared with abacavir hypersensitivity is in line with findings suggesting that direct interaction of flucloxacillin with the HLA-B*57:01 gene product is unlikely.(22) There is also *in vitro* data indicating covalent binding of flucloxacillin to cellular proteins during the T cell activation process, in contrast to what is believed to occur during activation with abacavir.(7, 22) These studies also suggested that a T cell response involving *HLA-B*58:01* might occur in flucloxacillin DILI, though the effect was less convincing that for B*57:01.(7) The B*58:01 protein sequence does not include V^{97} with Arg present instead at this position. No *HLA-B*58:01* positive carriers were found in our flucloxacillin cases. Since this allele is approx. 10 times more common in Europeans than *B*57:03*, we believe our study was powered adequately to detect this if present and we did detect it in our controls (frequency 0.03%). *B*58:01* did show an increased frequency compared with controls among the amoxicillin DILI cases so it appears to be a possible separate risk factor for DILI with this drug. HLA-*B*57:01* carriage also appears to be a risk factor for DILI due to pazopanib, which does not appear to have any structural homology to flucloxacillin or abacavir, though the effect size is much smaller than that observed for flucloxacillin.(23)

We also detected significant associations with additional HLA alleles and haplotypes. The association with the B57 haplotype demonstrated the clear specific association with *B*57:01*. Novel protective associations involving various alleles including *HLA-DQA1*03:01, HLA-C*07:02, HLA-B*07:02* and *HLA-DRB1*04:01* were seen. This protective effect for *HLA-B*07:02* is also detectable at the amino acid level with S^{97} in HLA-B associated with significant protection together with R^{97} which is the most common amino acid in this position in Europeans. Protective HLA associations have generally not been reported previously for DILI, except for one report of a protective HLA class II genotype against amoxicillin-clavulanate DILI(24) (25). *DRB1*04:01* is a well-established risk factor for autoimmune hepatitis,(26) so the protective effect observed here against flucloxacillin DILI is interesting.

The previous study on flucloxacillin DILI was relatively small and the number of cases that were *HLA-B*57:01*-negative was low.(6) In this larger study population, the frequency of *HLA-B*57:01* carriage of 82% is comparable to that in the original study and it seems likely that there is a genuine subgroup of cases (a total of 34 when two cases positive for the related *B*57:03* are excluded) that are not positive either for *HLA-B*57:01* or a closely related allele. This is supported by both the similarly in phenotype between the two groups and the fact that rigorous adjudication of cases was performed. We therefore examined genetic risk factors for this group in more detail, especially the possibility that the DILI reaction in these individuals might relate to penicillins more generally, since widely used penicillins such as amoxicillin occasionally give rise to DILI reactions (27-29) and we had positively adjudicated cases relating to both amoxicillin and other isoxazolyl penicillins available for study. GWAS analysis involving either the *B*57:01*-negative cases only or the combined group of *HLA-B*57:01* cases and any other penicillin (excluding amoxicillin-clavulanate) did not yield any genome-wide significant signals though this could reflect the small patient group. In view of the strong biological plausibility for a HLA association, we examined imputed HLA genotypes in detail with some additional direct typing. The B*57-negative flucloxacillin cases show significantly increased frequencies for several HLA class I alleles, but, these are seen at low frequencies in Northern Europeans (e.g. *HLA-A*02:02* has a frequency of 0.0008 in Germans and *A*30:01* is seen at 0.016). The pattern of HLA alleles seen in the amoxicillin DILI cases was not similar to that seen in either the B*57-negative flucloxacillin cases or the other isoxazolyl penicillin cases, suggesting that there is not a general risk factor which relates T cell responses to common structural features of these penicillins, at least *in vivo*. In line with a number of previous GWAS showing HLA associations for DILI(30, 31) and other adverse drug reactions(32) where small numbers of cases comparable in number to those in the current amoxicillin DILI group were studied, we believe *B*57:01* is not a risk factor for this form of DILI. However, interestingly, one of the HLA associations seen for the amoxicillin DILI group is with *B*58:01* which is closely related to *B*57:01* and strongly associated with cutaneous hypersensitivity reactions induced by allopurinol.(33) As mentioned previously, we believe that sufficient isoxazolyl penicillin cases including flucloxacillin cases were available to us to demonstrate no flucloxacillin DILI association with *B*58:01*, again suggesting the absence of a common "penicillin DILI" risk factor. In line with this, the structure of the side chain on a penicillin is generally considered the major determinant of T cell response(34) though cross-reactivity between some T cell clones in response to flucloxacillin and other isoxazolyl penicillins as well as amoxicillin has been demonstrated.(7) Therefore, the question of overall specificity is still not completely clear.

The finding that *B*57:01* was not common among the other isoxazolyl penicillin DILI group is interesting, especially since it is considered that, despite a strong structural homology to flucloxacillin, these beta lactamase-resistant penicillins are associated with a lower rate of DILI.(1, 5) Since patient numbers were small, this finding still needs to be treated with caution and should be investigated further. Estimates for rates of DILI due to non-flucloxacillin isoxazolyl penicillins in Iceland (where flucloxacillin is not licensed) are 1 in 26000 approx. compared with 1 in 12000 reported for first time users of flucloxacillin in the UK,(2, 35) though as with the genetic study this conclusion is based on small numbers of patients and prescriptions. *In vitro* studies also generally failed to show differences between the isoxazolyl penicillins in terms of T-cell interaction.(7) It is possible that the lower rate of DILI with these drug such as dicloxacillin and cloxacillin could partly reflect pharmacokinetic differences,(36) but, the failure to see any significant increased B*57-carriage in the cases that were available to us indicates that replacing the fluoride in flucloxacillin with a chloride or other group may change the structure of the putative peptide-drug complex sufficiently to eliminate initial presentation by B*57.

The association between *HLA-B*57:01* and flucloxacillin DILI remains the most significant report of a genetic association for DILI. General implementation of *B*57:01* testing in the clinic prior to flucloxacillin prescription appears impractical at present, but in the era of stratified medicine it seems possible that data on patient genotypes may become more generally available to prescribers. Using such data with other risk factors such as age and gender, especially in view of recent pharmacovigilance data showing increased risk above age 70,(2) could be a useful means of decreasing the incidence of flucloxacillin DILI in the future. Our current findings, which are also consistent with these age and gender effects,(2) suggest that additional genetic risk factors for flucloxacillin DILI are unlikely to be discovered in Europeans and that, despite the strong *B*57:01/03* association, approx. 20% of DILI cases relating to flucloxacillin would not be reliably predictable on the basis of carriage of this allele or any other common HLA allele.

A limitation of the current study is that we have predicted and not directly sequenced HLA alleles. The cost of sequencing and sample availability are barriers to direct HLA typing on all cases. HLA imputation is standard practice for research grade HLA analysis and the accuracy is consistently high, especially for HLA class I alleles, as shown in detail previously.(37) However, accuracy for rare allele assignment remains limited, so we cannot exclude the possibility that the flucloxacillin DILI cases might include additional extremely rare alleles positive for V^{97}.

In conclusion, this study has detected a novel association between *HLA-B*57:03* and flucloxacillin DILI in addition to the *HLA-B*57:01* association detected previously and confirmed that approximately 20% of the flucloxacillin DILI cases studied do not show any *B*57* association. The apparently lower risk for development of DILI with isoxazolyl penicillins other than flucloxacillin reported previously by others (1, 5) is consistent with our failure to see an association with *B*57:01* and other B*57 alleles for DILI due to these drugs. Other alleles which code for a HLA protein positive for V^{97} could be risk factors for flucloxacillin DILI in non-Europeans. It also remains possible that rare variants in HLA and other genes not covered by the GWAS chip or imputation may contribute to the risk of DILI with flucloxacillin and the other penicillins, but, population effect sizes from these seem unlikely to be large though for individuals the impact of rare variants may be higher.

Materials and methods

Enrollment details

All participants provided written informed consent and each study had been approved by the appropriate national or institutional ethical review boards. In the United Kingdom, ethical approval was via the Leeds East Research Ethics committee (approval reference 04/Q1206/91).

Causality assessment

The iDILIC cases were evaluated by application of the Council for International Organizations of Medical Science (CIOMS) scale, also called the Roussel Uclaf Causality Assessment Method (RUCAM)(11) and by expert review by a panel of three hepatologists. The pattern of liver injury was classified according to the International Consensus Meeting Criteria.(38) Only cases having at least a possible causality (score ≥3) were included in the study. For DILIN cases, causality assessment was by expert consensus as previously described.(12)

DNA preparation and Genome-wide genotyping of Phase II cases

For iDILIC cases, DNA was prepared as described previously.(6) DILIN DNA was extracted from lymphocytes and stored at the NIDDK biosample repository at Rutgers University, Piscataway, NJ.

Genome-wide genotyping of the phase II cases was performed by the Broad Institute, Boston on the Illumina Infinium HumanCoreExome BeadChip. iDILIC and DILIN cases were genotyped separately. Full details of the genotyping and quality control processes have been described previously.(12)

SNP and HLA Imputation

SNP imputation was performed as described recently.(12) Four digit HLA alleles and amino acid changes were also inferred using SNP2HLA using reference data collected by the Type 1 Diabetes Genetics Consortium (T1DGC).(17)

Direct HLA genotyping

Samples from phase I were genotyped for *HLA-B*57* using *HLA-B*57:01* genotyping with the Dynal AllSet Gold SSP B17 high resolution kit (Invitrogen). Selected HLA-*B*57:01* negative flucloxacillin DILI cases (n=33) were genotyped for all HLA-B alleles using an AllSet^{+}™ Gold sequence-specific primer (SSP) HLA-B Locus High Res Kit (Invitrogen) according to the manufacturer's instructions. Following PCR, products were applied to 2% agarose gels containing ethidium bromide (0.5 µg/ml) and electrophoresis was performed in 1 X TBE buffer. Positive lane amplifications were identified. HLA-B alleles were assigned by analysis with UniMatch® PLUS 6.0 SSP software (Invitrogen).

Statistical analysis

All genetic analyses were performed as described previously.(12) For HLA analysis, we tested for association of carriage of each HLA allele/AA/HLA haplotype. MHC significance was defined using the Bonferroni correction threshold of P<0.00025 (0.05/200 accounting for 200 observed HLA alleles). Conditional analyses in the MHC region were undertaken and the genotypes at the conditioning SNP(s) were included as covariates under an additive model. For clinical variable comparisons, Fisher's exact test was used for categorical covariates such as gender and pattern of liver damage. Univariate and multivariate analysis for studies combining genetic and clinical risk factors was performed by STATA15.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Flucloxacillin is known to be a common cause of drug-induced liver injury. Previously, carriage of *HLA-B*57:01* has been demonstrated to be a strong genetic risk factor for this adverse drug reaction in a small genome-wide association study.

WHAT QUESTION DID THIS STUDY ADDRESS?

To extend the previously reported *HLA-B*57:01* association in an enlarged cohort, identify additional genetic factors and assess the relevance of risk factors for flucloxacillin-related liver injury to liver injury with other penicillins, both other isoxazoyl penicillins such as dicloxacillin and also amoxicillin.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

We found that a related allele *HLA-B*57:03* was also a risk factor and that the amino acid valine at position 97 which is common to both B*57:01 and B*57:03 HLA proteins was the key risk factor at the amino acid level. We detected no *HLA-B*57* association with liver injury due to other isoxazolyl penicillins or amoxicillin and no significant non-HLA signals for any penicillin-related liver injury.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Prior knowledge of *HLA-B*57* genotype may affect the decision to prescribe flucloxacillin especially in patients aged 70 years and older who have an increased risk of liver injury with this drug.

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GPA, EB, RJA; sample preparation and laboratory analysis: SAC, TC and MA; data analysis and interpretation: PN, YS, JIG, GPA, MRN and AKD; writing the manuscript: PN, GPA and AKD

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Table 1 Summary of clinical data for the flucloxacillin DILI cases

Number of cases	197
Gender (F/M)	133/64 (68% female)
Mean Age at onset (years) (with standard deviation)	62 ± 13
Mean Time to onset (days) (with standard deviation)	24 ± 18
Total days on drug (with standard deviation)	10 ± 6
Pattern of liver injury	
Cholestatic	74 (38%)
Hepatocellular	39 (20%)
Mixed	84 (43%)
Scoring (CIOMS/RUCAM)	
3-5 (possible)	22 (11%)
6-8 (probable)	90 (46%)
>8 (highly probable)	85 (43%)

Table 2 The most significant HLA alleles for flucloxacillin DILI

ALLELE	OR	95% CI	P	Cond OR	Cond P	AF cases	AF controls
*B*57:01*	36.62	26.14-51.29	2.6×10^{-97}	-	-	0.42	0.04
*C*06:02*	10.11	7.88-12.97	4.3×10^{-74}	1.32	0.23	0.45	0.09
*DQB1*03:03*	10.18	7.77-13.34	1.1×10^{-63}	0.96	0.84	0.31	0.05
*DRB1*07:01*	4.02	3.23-5.02	3.8×10^{-35}	1.01	0.94	0.38	0.13
*DQA1*02:01*	4.02	3.22-5.01	4.5×10^{-35}	1.01	0.95	0.38	0.13
*A*01:01*	1.86	1.5-2.31	1.8×10^{-8}	0.95	0.69	0.30	0.18
*DQA1*03:01*	0.42	0.3-0.58	3.0×10^{-7}	0.61	0.009	0.10	0.21
*C*07:02*	0.33	0.22-0.51	4.2×10^{-7}	0.63	0.04	0.06	0.16
*B*07:02*	0.32	0.2-0.5	5.7×10^{-7}	0.60	0.04	0.05	0.15
*DQB1*03:01*	0.51	0.36-0.71	7.3×10^{-5}	0.76	0.14	0.10	0.17
*DQB1*06:02*	0.46	0.31-0.69	0.0001	0.69	0.09	0.07	0.14
*DRB1*04:01*	0.46	0.3-0.7	0.0003	0.67	0.09	0.06	0.12
*DQB1*03:02*	0.47	0.3-0.73	0.0007	0.68	0.11	0.05	0.11
*B*57:03*	19.77	3.37-116.1	0.001	79.21	0.000001	0.005	0.0003

OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value; Cond OR = Odds Ratio from the reciprocal conditional analyses on rs2395029 and *HLA-B*57:01*; Cond P = logistic p-value from the conditional analysis; AF cases = allele frequency in cases; AF controls = allele frequency in controls. The p value required for significance was P<0.00025.

Table 3 Effect size of the imputed amino acid residues located at position 97 on HLA-B gene product

Residues	OR	95% CI	P	AF cases	AF controls
Valine (V)	38.10	27.07-53.62	9.7×10^{-97}	0.43	0.04
Arginine (R)	0.43	0.34-0.53	5.13×10^{-14}	0.28	0.48
Serine (S)	0.53	0.41-0.68	9.82×10^{-7}	0.18	0.30
Asparagine (N)	0.31	0.14-0.69	0.004	0.02	0.05
Threonine (T)	0.64	0.42-0.96	0.03	0.06	0.10
Tryptophan (W)	0.87	0.5-1.54	0.64	0.03	0.04

Residue = name of the associated amino acid at position 97; OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value; AF cases = allele frequency in cases ; AF controls = allele frequency in controls. The significance threshold was $p<0.00025$.

Table 4 HLA allele frequency data for the *HLA-B*57:01* and *HLA-B*57:03* negative flucloxacillin DILI cases

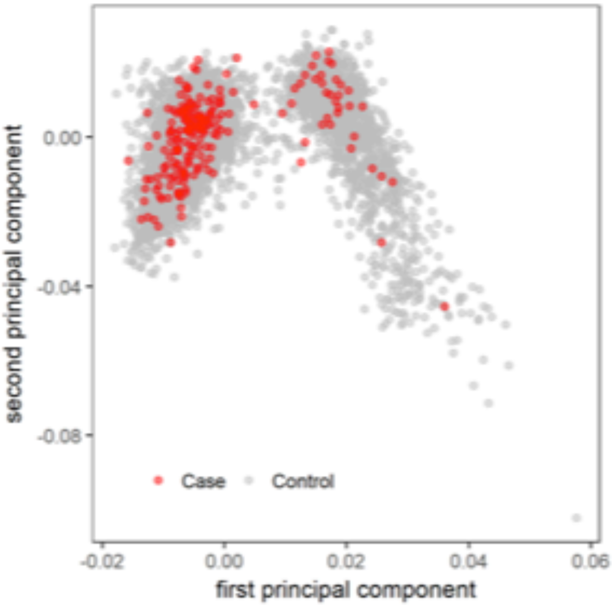
ALLELE	OR	95% CI	P	AF cases	AF controls
*HLA-A*02:02*	15.24	1.89-123.1	0.01	0.01	0.001
*HLA-A*30:01*	4.447	1.38-14.34	0.01	0.04	0.01
*HLA-B*13:02*	3.496	1.22-10.06	0.02	0.06	0.02
*HLA-DQB1*03:02*	0.1228	0.02-0.89	0.04	0.01	0.11
*HLA-B*07:02*	0.347	0.13-0.95	0.04	0.06	0.15

OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value; AF cases = allele frequency in cases; AF controls = allele frequency in controls. The p value required for significance was $P<0.00025$.

Table 5 HLA associations across different penicillin classes

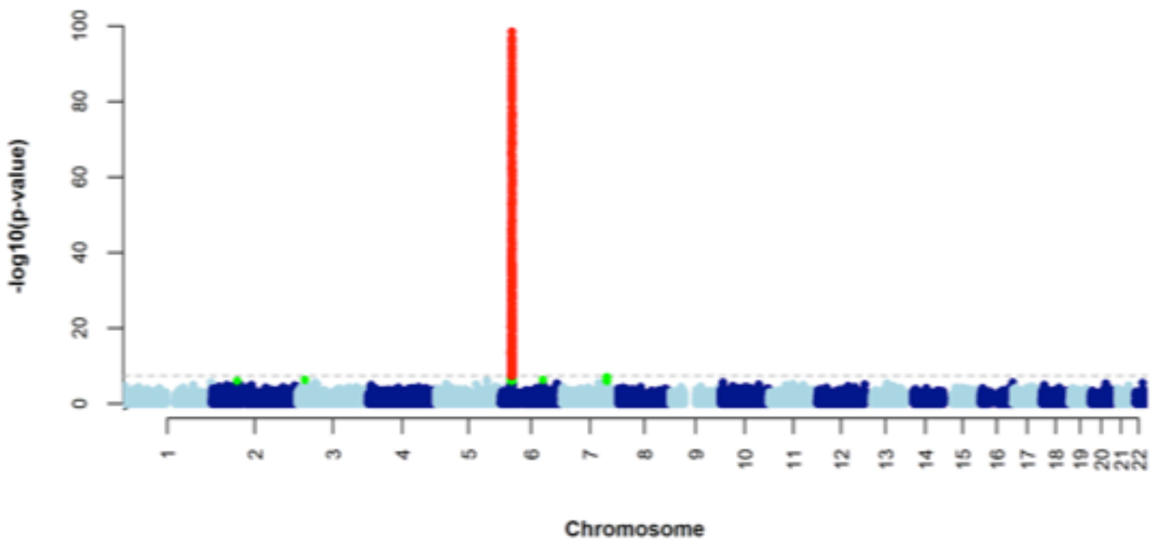
Group	HLA Allele	OR	95% CI	P	AFcases	AF controls
Other isoxazolyl penicillins (n=6)	*C*07:04*	12.97	1.58-134.6	0.001	0.17	0.02
	*DQB1*06:09*	14.57	2.18-12.16	0.02	0.08	0.01
Amoxicillin (n=15)	*B*58:01*	20.68	4.31-99.12	0.0002	0.07	0.01
	*DPB1*01:01*	4.84	2.07-11.32	0.0003	0.233	0.054
	*A*01:01*	3.25	1.59-6.62	0.001	0.433	0.159
	*C*03:02*	30.09	3.55-255.2	0.002	0.033	0.002

OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value; AF cases = allele frequency in cases ; AF controls = allele frequency in controls. The p value required for significance was $P<0.00025$.



second principal component
0.00
-0.04
-0.08
Case
Control
-0.02
0.00
0.02
0.04
0.06
first principal component

Figure 1 Scatterplot representing the case control distribution of study cohort. The axes represent the first two principal components where the red dots are the cases and the gray dots the controls. The controls cluster in two groups representing the UK and Swedish major control populations.


-log10(p-value)
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Chromosome

Figure 2 Manhattan plot from the GWAS analysis of 197 flucloxacillin cases and 6835 controls. Manhattan plot displays the negative log of the significance for each tested variant ranked by chromosomes and position; SNPs in green have a significance level less than $5x10^{-6}$ and red have a significance level less than $5x10^{-8}$ which was taken as the threshold for genome-wide significance.

Supplementary Information Titles

Supplied as single PDF file with the following content:

iDILIC investigators

Supplementary Tables 1-8

Supplementary Figures 1-5

Supplementary Material for "Drug-induced injury due to flucloxacillin: relevance of multiple HLA alleles"

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Table S1

Clinical information for other non-flucloxacillin DILI cases

Clinical information	**Amoxicillin**	**Isoxazolyl penicillins**
Total number of cases	15	6
Gender (F/M)	9/6 (57%)	3/3 (50%)
Mean Age at onset (years) (with standard deviation)	60 ±19	60 ±25
Mean Time to onset (days) (with standard deviation)	25 ±22	18 ±10
Total days on drug (with standard deviation)	13 ±14	17± 12
Pattern of liver injury		
Cholestatic	9	2
Hepatocellular	5	2
Mixed	1	1
Unknown	-	1
CIOMS/RUCAM scoring		
3-5 (possible)	2	-
6-8 (probable)	10	4
>8 (highly probable)	3	2

Table S2

Haplotype association results for flucloxacillin DILI using the six HLA alleles belonging to B57 haplotype

	FREQ in cases	FREQ in Controls	OR	P
Positive B*57:01 haplotypes				
PPPPPA	0.15	0.013	18.7	6.19E-59
PPPPPP	0.14	0.013	18.6	9.12E-53
PPAAAA	0.06	0.005	15.4	7.45E-24
PPAAAP	0.04	0.004	16.1	5.55E-19
PAPPPP	0.01	0.0003	61.5	7.27E-08
PPAPPA	0.009	0.0007	36.8	4.66E-06
PAPPPA	0.004	0.0006	7.65	0.03
PPAPPP	0.002	0.0003	17.4	0.07
PAAAAP	0.0019	0.0006	4.34	0.3
PAAAAA	0.0009	0.0006	1.53	0.8
PAAPPA	0	0.0003	4.37E-190	1.0
PAAPPP	0	0.0001	3.17E-129	1
PPAPAP	0.002	0	3.45E+11	1
PPPAAP	0	3.19E-05	2.07E-52	1
PPPAAA	0	3.62E-06	1.94E-157	1
PAPPAA	0	0	0	0
PAAPAA	0	0	0	0
Negative B*57:01 haplotypes				
AAAAAA	0.40	0.6739	0.278	2.39E-27
AAAAAP	0.09	0.1401	0.578	0.003
AAAPPA	0.04	0.06	0.482	0.01
AAAPPP	0.001	0.006	0.00224	0.06
AAPAAA	0.003	0.01326	0.147	0.1
AAPPPA	0.0030	0.0083	0.267	0.2
APPPPP	0.0013	0.0005	5.15	0.4
AAPPPP	0.0002	0.0016	0.003	0.4
AAPAAP	0.0001	0.0011	4.92E-06	0.5
APAPPP	0.0018	0.0031	0.296	0.5
APAAAA	0.0150	0.0196	0.745	0.5
APAAAP	0.0073	0.0088	0.776	0.7

AAAPAA	0.0001	0.0003	0.24	0.8
APPPPA	0.0003	0.0005	0.335	0.8
AAAPAP	4.95E-05	0.0002	0.237	0.9
APAPPA	0.02	0.02	0.96	0.9
AAAAPA	0	0.0001		1.0
APPAPP	0	5.96E-05		1
APPAAP	0	2.97E-05		1
AAPAPA	0	1.20E-05		1
APAPAA	0	2.91E-05		1
APPAAA	0	2.72E-05		1
AAPPAA	0	0	0	0

Odds ratios (OR) and p-values (P) are presented after correcting for population stratification for each combination of alleles belonging to B57 haplotype. The six letters in the first column (haplotype) reflects in order of HLA-B*57:01|HLA-C*06:02|HLA-DQB1*03:03|HLA-DRB1*07:01|HLA-DQA1*02:01|HLA-A*01:01 status. The risk alleles status is represented by P = present or A = absent.

Table S3

HLA alleles which share valine at position 97

ALL	MAF	Dataset
B*57:01	0.04	USA NMDP European Caucasian
B*57:03	0.0007	USA NMDP European Caucasian
B*57:10	0.0003	Poland DKMS
B*57:02	0.0002	USA NMDP European Caucasian
B*57:07	0.00001	USA NMDP European Caucasian
B*57:04	0.000003	USA NMDP European Caucasian
B*40:30	0.0000008	USA NMDP European Caucasian
B*40:34	0.0000008	USA NMDP European Caucasian
B*57:14	0.0000008	USA NMDP European Caucasian
B*57:16	0.0000008	
B*57:08	0.0000004	USA NMDP European Caucasian
B*57:15	0.0000004	USA NMDP European Caucasian
A*26:32	0	
A*30:28	0	
B*55:14	0	
B*57:06	0	USA NMDP European Caucasian
B*57:09	0	
B*57:12	0	
B*57:13	0	
B*57:17	0	
B*57:18	0	
B*57:19	0	
B*58:14	0	

Table S4. Case-control analysis on effect of age, gender and B*57 genotype on risk of flucloxacillin DILI

Univariate analysis			
FACTOR	**OR**	**95% CI**	**P**
age	1.09	1.06-1.10	7.1×10^{-20}
>70 years	6.27	3.75-10.50	2.7×10^{-12}
Gender (Female)	1.69	1.14-2.36	4.2×10^{-3}
HLA B*57 allele	43.09	24.19-76.74	2.1×10^{-37}
Multivariate analysis			
FACTOR	**OR**	**95% CI**	**P**
Gender (Female)	1.66	0.92-3.00	9.1×10^{-2}
>70 years	6.70	3.07-15.02	2.2×10^{-6}
HLA B*57 allele	42.45	23.04-78.14	2.5×10^{-33}

Age is analyzed as a continuous variable in the univariate analysis

Table S5. Comparison of selected parameters between HLA-B*57-positive and negative cases relating to flucloxacillin

	B*57 positive (n=163)	B*57 negative (n=34)
Gender (F/M)	114F/49M (70% female)	19F/15M (56% female)
Age at onset (years) (mean ±SD)	63±13	61±10
Time to onset (days) (mean ±SD)	23±13	30±30
Total days on drug (mean ±SD)	10±5	9±7
Pattern of liver injury		
Cholestatic	63	11
Hepatocellular	31	8
Mixed	69	17
Scoring (CIOMS/RUCAM)		
3-5 (possible)	18	4
6-8 (probable)	73	17
>8 (highly probable)	72	13

There were no statistically significant differences between the two groups for the parameters listed

Table S6. The most associated amino acid deletions in the HLA-B*57:01-negative flucloxacillin case analysis

POSITION OF DELETED RESIDUE IN HLA-A	OR	95%CI	P
276	17.85	2.16-147.4	0.007
282	17.85	2.16-147.4	0.007
283	17.85	2.16-147.4	0.007
288	17.85	2.16-147.4	0.007
294	17.85	2.16-147.4	0.007
297	17.85	2.16-147.4	0.007
298	17.85	2.16-147.4	0.007
299	17.85	2.16-147.4	0.007
307	17.85	2.16-147.4	0.007
310	17.85	2.16-147.4	0.007
311	17.85	2.16-147.4	0.007
321	17.14	2.07-141.6	0.008
334	17.14	2.07-141.6	0.008
-11	16.55	2.02-135.4	0.009
-15	16.55	2.02-135.4	0.009
-20	16.55	2.02-135.4	0.009
-22	16.55	2.02-135.4	0.009

Residue in HLA-A= Amino acid position along the HLA-A protein product; OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value

Table S7 Effect size of the HLA-B*57:01 association across different drug groups

DRUG GROUP	OR	95%CI	PV	AF_{cases}
Flucloxacillin	36.62	26.14-51.29	$2.6x10^{-97}$	0.48
Amoxicillin	2.80	0.84-9.42	0.09	0.1
Other isoxazolyl penicillins	3.09	0.37-25.78	0.28	0.1

OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value; AF cases = allele frequency in cases.

Table S8

The most associated amino acids in the analysis on DILI due to penicillins other than flucloxacillin

DRUG GROUP	LOCUS & POSITION	AMINO ACID	OR	95% CI	P
Isoxazolyl penicillins (n=6)	HLA-C (95)	Phenylalanine	12.97	2.69-62.64	0.001
	HLA-C (156)	Aspartic acid	12.97	2.69-62.64	0.001
Amoxicillin (n=15)	HLA-DPB1 (194)	Glutamine	4.86	2.08-11.35	0.0003
	HLA-B(67)	Phenylalanine /Methionine	3.55	1.7-7.41	0.0008
	HLA-A(44)	Lysine	3.23	1.59-6.57	0.0012
	HLA-A(67)	Methionine	3.23	1.59-6.57	0.0012

OR = Odds Ratio; 95% CI = 95% Confidence Interval of the odds ratio; P = logistic p-value

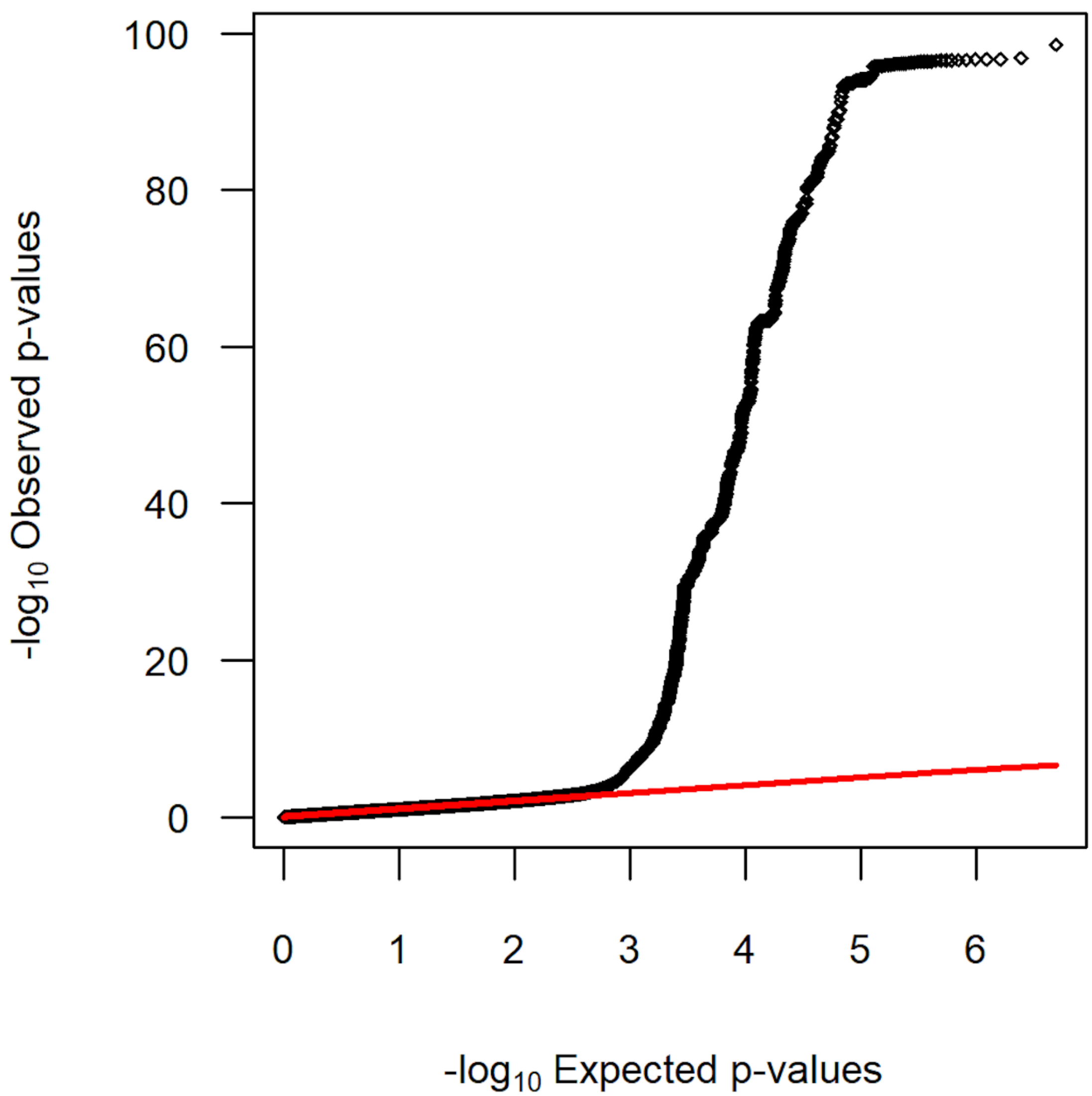
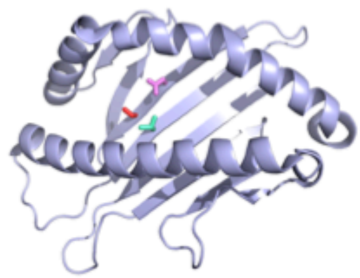


Figure S1. QQ plot for the main Flucloxacillin GWAS.



```
                   100        110        120
B*07:02 GSHTLQSMYG CDVGPDGRLL RGHDQYAYDG
B*57:01 GSHIIQVMYG CDVGPDGRLL RGHDQSAYDG
B*57:02 GSHIIQVMYG CDVGPDGRLL RGHNQYAYDG
B*57:03 GSHIIQVMYG CDVGPDGRLL RGHNQYAYDG
```

Figure S2. Molecular structure of the HLA-B*57:01 antigen binding cleft with key amino acids contributing to abacavir binding marked. The structure shown is an illustration of HLA-B*57:01 visualised in PyMOL (Version 1.2b2; http://www.pymol.org) based on the Illing structure (3VRI)(Illing, P.T. *et al.* Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature* **486**, 554-8 (2012)) available from Protein Data Bank. Residue 97 (valine) is highlighted in green, 114 (aspartic acid) in pink and 116 (serine) in red. Alignment of amino acids in the region 90 to 120 is shown for B*57:01, B*57:02 and B*57:03 with B*07:01, the most common B allele in European populations as reference. Key amino acid differences are highlighted.

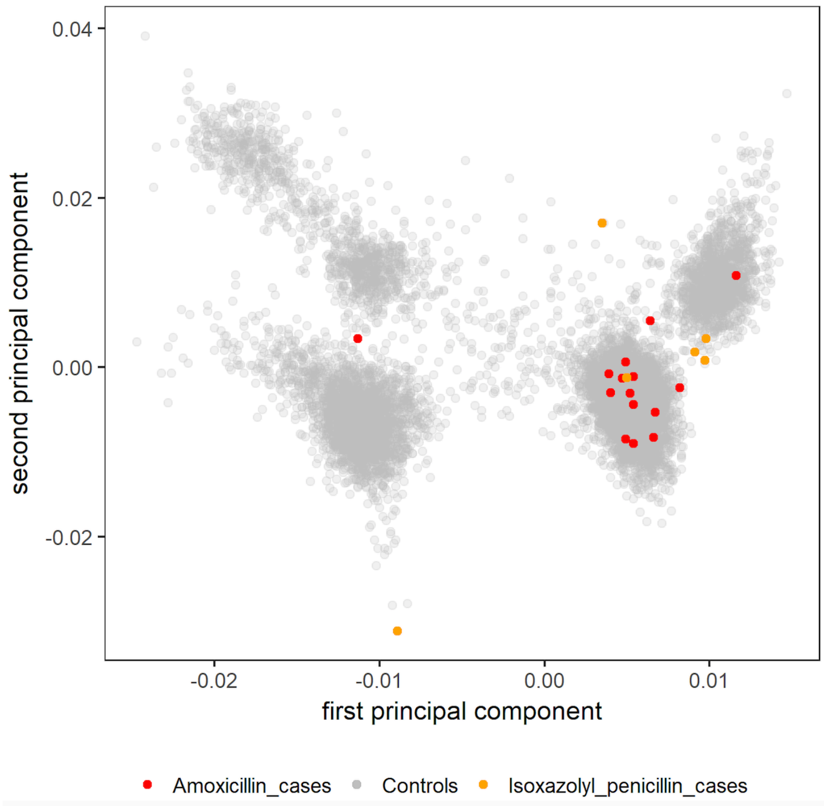


Figure S3. Scatterplots representing the first two principal components of the European study cohort. Amoxicillin cases are highlighted in red and the isoxazolyl penicillin cases in orange while controls are highlighted in gray.

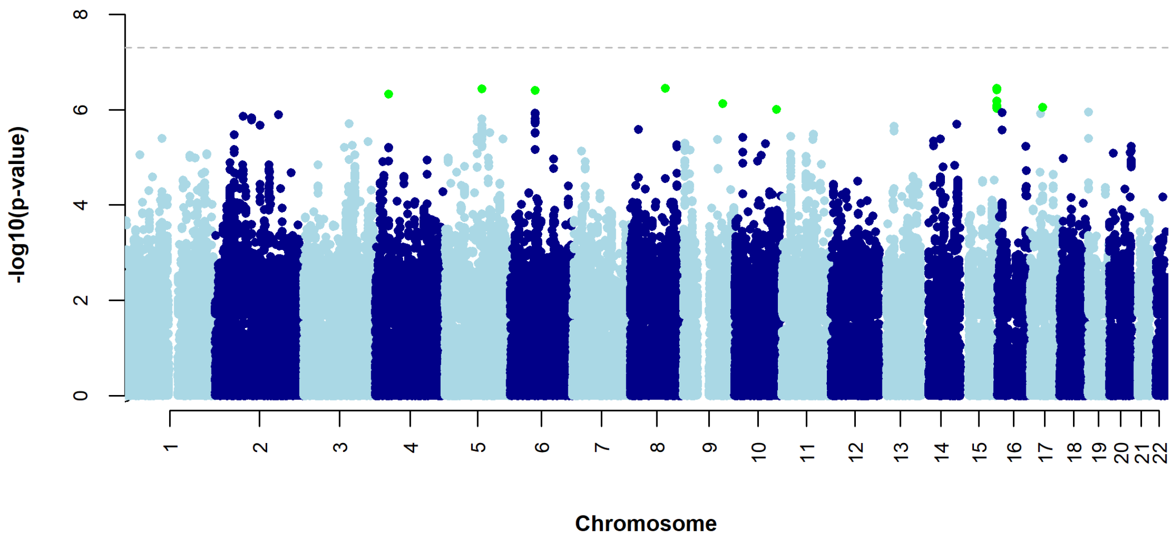


Figure S4. Manhattan plot from the GWAS analysis of 6 isoxazolyl penicillin cases and 10588 controls. Manhattan plot displays the negative log of the significance for each tested variant ranked by chromosomes and position; SNPs in green have a significance level less than $5x10^{-6}$ and red have a significance level less than $5x10^{-8}$.

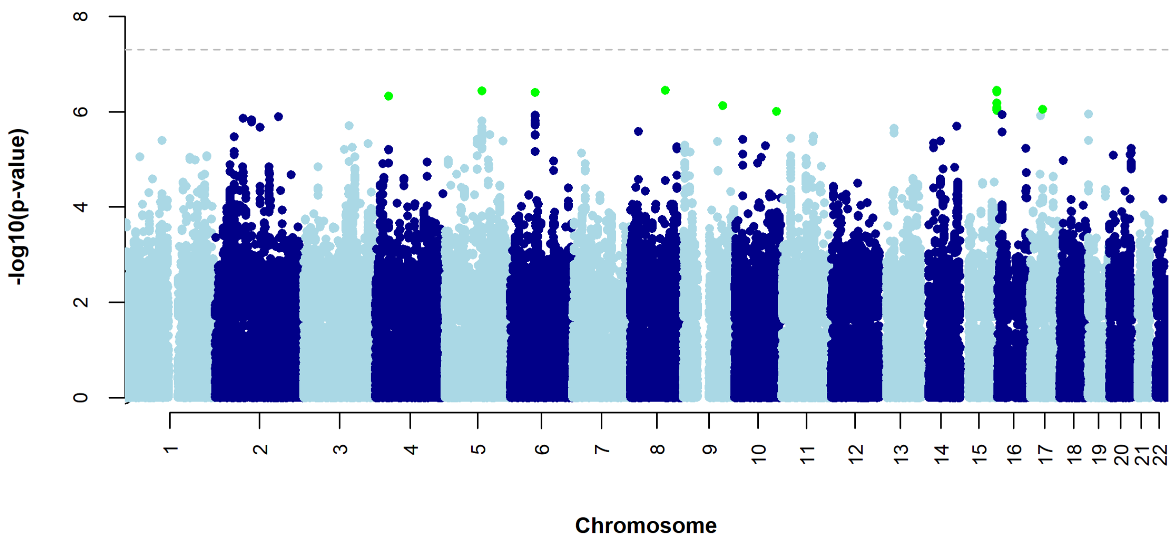


Figure S5. Manhattan plot from the GWAS analysis of 15 amoxicillin cases and 10588 controls. Manhattan plot displays the negative log of the significance for each tested variant ranked by chromosomes and position; SNPs in green have a significance level less than $5x10^{-6}$ and red have a significance level less than $5x10^{-8}$.